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A Bartter's Syndrome Mutation of ROMK1 Exerts Dominant Negative Effects on K^+ Conductance

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Key Words

ROMK1 • Kir 1.1 • Bartter's syndrome type 2 • Mutation • K^+ channel • *Xenopus* oocytes

Abstract

Mutations in the gene encoding the renal epithelial K^+ channel ROMK1 (Kir 1.1) is one of the causes for Bartter's syndrome, an autosomal recessive disease. It results in defective renal tubular transport in the thick ascending limb of the loop of Henle that leads to hypokalemic metabolic alkalosis and loss of salt. Two novel ROMK1 mutations, L220F/A156V, have been described recently in a compound heterozygote patient demonstrating typical manifestations of Bartter's syndrome. Functional properties of these ROMK1 mutants were studied by coexpressing in *Xenopus* oocytes and by means of double electrode voltage clamp experiments. When both ROMK1 mutants were coexpressed no K^+ conductance could be detected. The same was found in oocytes expressing A156V-ROMK1 only or coexpressing wild type (wt) ROMK1 together with A156V-ROMK1. In contrast, K^+ conductances were indistinguishable from that of wt-ROMK1 when L220F-ROMK1 was expressed alone. Activation of protein kinase C signaling inhibited the conductance in both L220F-ROMK1 and wt-ROMK1 expressing oocytes. These effects were not seen in A156V-ROMK1 expressing oocytes. Because no further abnormalities in the properties or regulation of L220F-ROMK1 were detected, we conclude that A156V-ROMK1 has a dominant negative effect on L220F-ROMK1 thereby causing Bartter's syndrome type two in this patient.

Introduction

Bartter's syndrome is an autosomal recessive disease affecting renal tubular absorption of NaCl in the thick ascending limb of the loop of Henle (TAL) and therefore leads to a loss of salt, hypokalemic metabolic alkalosis with high plasma renin and aldosterone concentrations. In some cases it may be associated with low blood pressure [1]. Mutations in three different genes have been identified as the cause for Bartter's syndrome including that of the furosemide – inhibited Na^+-$2Cl^-$-K^+ cotransporter, the K^+ channel ROMK1 in the luminal membrane of TAL and the chloride channel CLC-Kb in the basolateral membrane [2-7]. The renal K^+ channel ROMK1 is an inwardly

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rectifying ATP-regulated potassium channel that is regulated by intracellular pH [8;9]. It serves as an apical recycling pathway for K^+ ions as well as the K^+ secretory pathway in the distal tubule. It is therefore responsible for reabsorption of Na^+ in both tubular segments [8;10;11].

Several mutations in the ROMK1 gene have been identified which cause antenatal Bartter's syndrome [3;5;6]. Among them, there have been two novel mutations that were identified recently in a compound heterozygote patient. These mutations, L220F and A156V, led to the appearance of a typical neonatal Bartter's syndrome [5]. It was, however, not clear how these mutations affect K^+ channel function of ROMK1. The goal of the present report therefore is, to determine functional consequences of these novel Bartter's syndrome mutations. To that end, characteristic properties of wtROMK1 were assessed and compared with that of mutant ROMK1.

Materials and Methods

In vitro synthesis of mutant ROMK1

cDNA encoding human ROMK1 was kindly provided by Dr. C. Derst, Philipps - Universität Marburg, Germany. The mutations L220F and A156V were generated by polymerase chain reaction (PCR) using the following primers i) (L220F) 5´-GGC TAA TCT CAG GAA AAG CTT TCT TAT TGG CAG TCA-3´ (sense) and 5´-TGA CTG CCA ATA AGA AAG CTT TTC CTG AGA TTA GCC-3´ (antisense) generating a *Hind*III site; ii) (A156V) 5´-GAC AGA ACA GTG CGC AAC TGT CAT TTT TCT GCT T-3´ (sense) and 5´-AAG CAG AAA AAT GAC AGT TGC GCA CTG TTC TGT C-3´ (antisense) generating a *Mst*I site (QuikChange™, Stratagene). The methylated, nonmutated template was digested with 10 U *Dpn*I and transformed in XL1-Blue competent cells. Positive colonies were identified by *Hind*III (L220F) and *Mst*I (A156V) digest. Mutations were confirmed for correct codon sequences by dideoxynucleotide termination DNA sequencing (Thermo Sequenase I, Pharmacia) using an 373A DNA sequencer (Applied Biosystems).

Injection of ROMK1 – cRNA and electrophysiological analysis

cDNAs encoding wild type (wt) and mutant (A156V, L220F) ROMK1 were linearized using *Kpn*I and cRNA was *in vitro* transcribed using T3 or polymerases and a 5´cap (mCAP mRNA capping kit, Stratagene). Isolation and microinjection of oocytes have been described in a previous report [12]. In brief, after isolation from adult *Xenopus laevis* female frogs oocytes were dispersed and defolliculated by a 0.5 h treatment with collagenase (type A, Boehringer, Germany). Subsequently oocytes were rinsed and kept in ND96-buffer (in mmol/l): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, pH 7.55, supplemented with theophylline (0.5 mmol/l) and gentamycin (5 mg/l) at 18 °C. Oocytes of identical batches were injected with cRNA of either wt or mutant ROMK1 (each 10 ng, if not stated otherwise) dissolved in 50 nl double-distilled water (PV830 pneumatic pico pump, WPI, Germany). Oocytes injected with 50 nl double-distilled water served as controls. Three days after injection oocytes were impaled with two electrodes (Clark instruments) which had resistances of 1 MΩ when filled with 2.7 mol/l KCl. A flowing (2.7 mol/l) KCl electrode served as bath reference in order to minimize junction potentials. Membrane currents were measured by voltage clamping of the oocytes (OOC-1 amplifier, WPI, Germany) between -100 and +40 mV in steps of 20 mV, each lasting 1000 ms. Current data were filtered at 400 Hz (OOC-1 amplifier). Between intervals, oocytes were voltage clamped to their spontaneous membrane voltage for 20 s. Oocytes expressing either wt or mutant ROMK1 were measured at the same day of expression in identical batches of ooyctes. Data were collected continuously on a computer hard disc, displayed on a computer screen and analyzed using the programs chart and scope (McLab, AD-Instruments, Macintosh). Whole cell chord conductances were calculated for the positive (G_{Ipos}) and negative (G_{Ineg}) current range separately according to Ohm's law. During the entire experiment the bath was continuously perfused at a rate of 5 - 10 ml/min. All experiments were conducted at room temperature (22 °C). All used compounds were of highest available grade of purity. 3-isobutyl-1-methylxanthine (IBMX), forskolin, 1,2-dioctanoyl-sn-glycerol (DOG) and bisindolylmaleimide I (BIM) were all from Sigma (Deisenhofen, Germany). Ba^{2+} and TEA^+ were obtained from Merck (Darmstadt, Germany). Statistical analysis was performed according to Students t test. A p value <0.05 was accepted to indicate statistical significance.

Results

K^+ currents formed by expression of wt-ROMK1, L220F-ROMK1 and A156V-ROMK1

Expression of wt-ROMK1 induced a large inwardly rectifying K^+ current that was inhibited almost completely by application of 5 mmol/l Ba^{2+} and TEA^+ to the extracellular bath solution (Fig.1 A). The inhibitory effect was more pronounced at negative clamp when compared to positive clamp voltages, which probably reflects the fact that Ba^{2+}/TEA^+ find better access to the K^+ channel at negative voltages (Fig1. A,B,C). Due to K^+ channel expression the membrane voltage was significantly hyperpolarized (Fig. 1B). Ba^{2+} and TEA^+ shifted the zero current voltage to depolarized values.

Very similar results were obtained upon expression of L220F-ROMK1 (Fig.2 A,B). The summaries of the calculated whole cell conductances shown in Figs.1C and 2B indicate similar magnitudes of K^+ conductances generated by either wt-ROMK1 or L220F-ROMK1. However, when A156V-ROMK1 was expressed no whole cell K^+ current was detected and the calculated whole cell

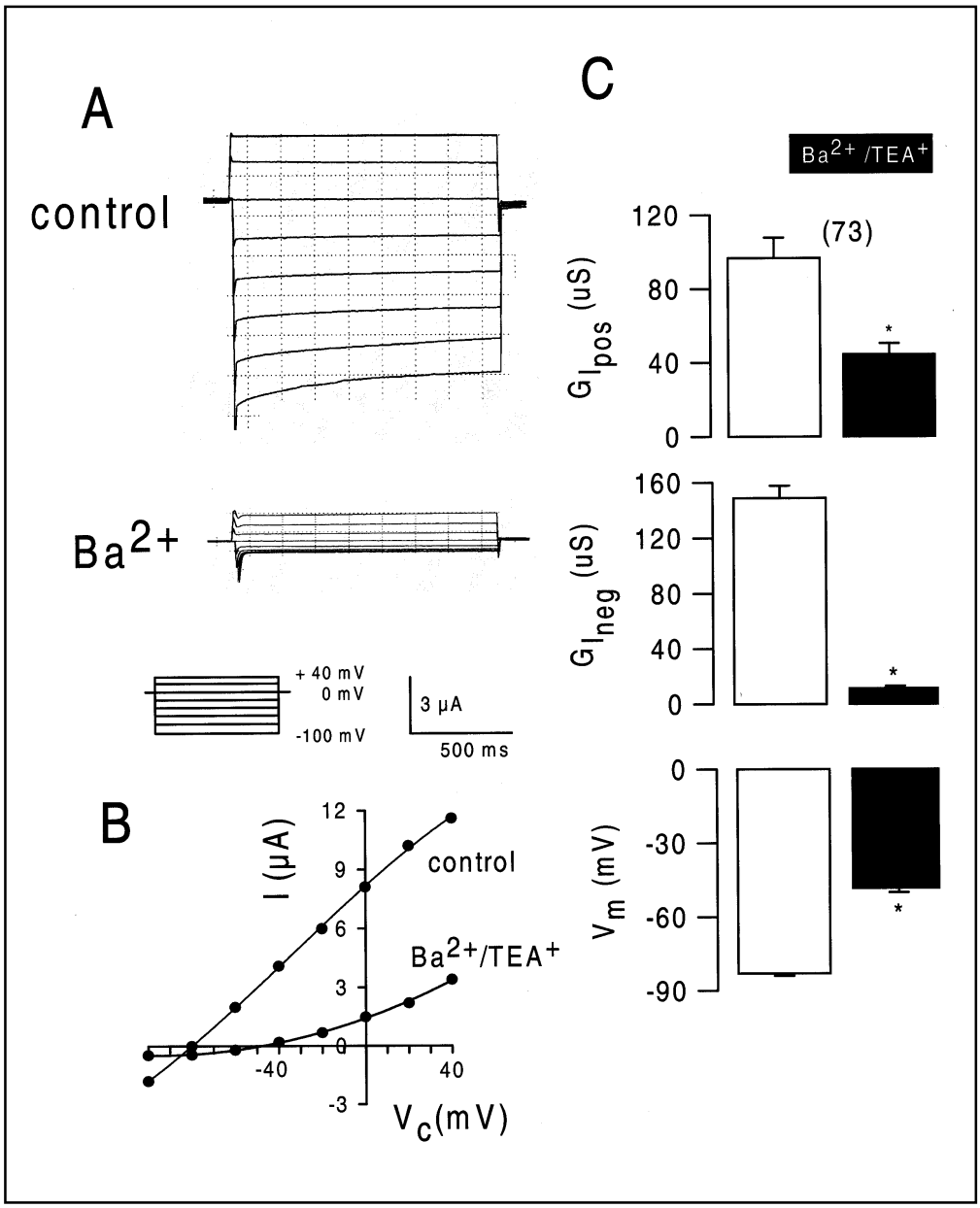

Fig. 1. ROMK1 whole cell currents in *Xenopus* oocytes. A) Representative example of whole cell currents produced by wt-ROMK1 before and after inhibition by Ba^{2+} and TEA^+ (5 mM each). B) Corresponding current (I) voltage (V_c) curves indicate more pronounced inhibition at negative clamp voltages due to better blockage by positively charged Ba^{2+} and TEA^+. C) Summary of the whole cell chord conductances, calculated for the positive (G_{Ipos}) and negative (G_{Ineg}) current range separately, and the membrane voltage measured before (white bars) and after (black bars) inhibition by Ba^{2+}/TEA^+. Means ± SEM (number of experiments). Asterisks indicate significant difference from control.

conductances in these oocytes were similar to those of water injected control oocytes. Moreover, injection of higher amounts of A156V-ROMK1 cRNA failed to induce K^+ currents. Accordingly, the membrane voltages of A156V-ROMK1 expressing oocytes were not different to that of water injected oocytes.

Protein kinase dependent regulation

We examined some characteristic properties of wt-ROMK1, L220F-ROMK1 and A156V-ROMK1. Stimulation of the oocytes by 1 mmol/l 3-isobutyl-1-methylxanthine (IBMX) and 1 µmol/l forskolin (activation of protein kinase A, PKA) had no effects on either wt-ROMK1 or L220F-ROMK1 conductance (Fig. 3). Moreover, no K^+ conductance was activated by IBMX/forskolin in ooyctes expressing A156V-ROMK1. When oocytes were incubated for 15 min in 5 µmol/l 1,2-dioctanoyl-sn-glycerol (DOG), an activator of protein kinase C (PKC), the whole cell conductances of both wt-ROMK1 and L220F-ROMK1 were inhibited slightly but significantly. In contrast, a 20 min incubation with 0.1 µmol/l bisindolylmaleimide I (BIM), an inhibitor of PKC,

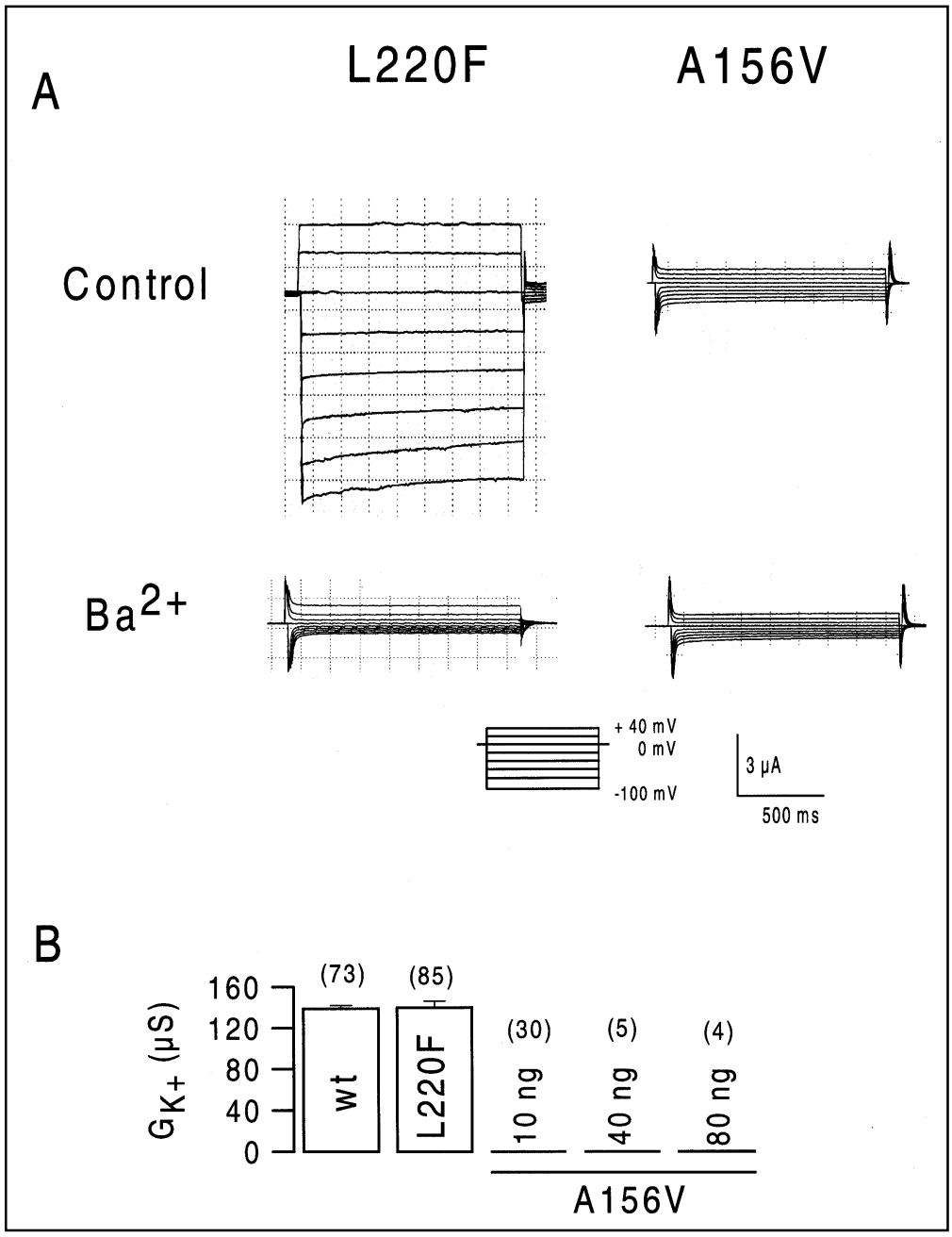

Fig. 2. Whole cell currents produced in *Xenopus* oocytes by expression of L220F-ROMK1 (left) and A156V-ROMK1 (right). A) Representative example of whole cell currents before and after inhibition by Ba^{2+} and TEA^{+} (5 mM each). B) Summary of the Ba^{2+}/TEA^{+} - sensitive whole cell conductances (negative current range; G_{K+}) induced by expression of wt-ROMK1, L220F-ROMK1 and after injection of various amounts of cRNA of A156V-ROMK1. Means ± SEM (number of experiments). Asterisks indicate significant difference from control.

had the opposite effects: the whole cell conductances in both, oocytes expressing wt-ROMK1 or L220F-ROMK1, were significantly enhanced (Fig. 3). No effects of BIM were observed for A156V-ROMK1 expressing oocytes. Finally, oocytes were bathed in 3 mmol/l butyrate (pH 6.5) in order to acidify the cytosolic compartment. This maneuver induced comparable inhibition of K^{+} conductances in wt-ROMK1 and L220F-ROMK1 expressing oocytes, but had no effects on A156V-ROMK1 injected oocytes. These data demonstrate identical properties for wt-ROMK1 and L220F-ROMK1 and indicate that A156V-ROMK1 cannot be activated by either PKA or inhibition of PKC.

Dominant negative effects of A156V-ROMK1

Because the mutation L220F did not affect ROMK1 in its ability to function as a K^{+} channel when expressed in *Xenopus* oocytes, we examined possible effects of

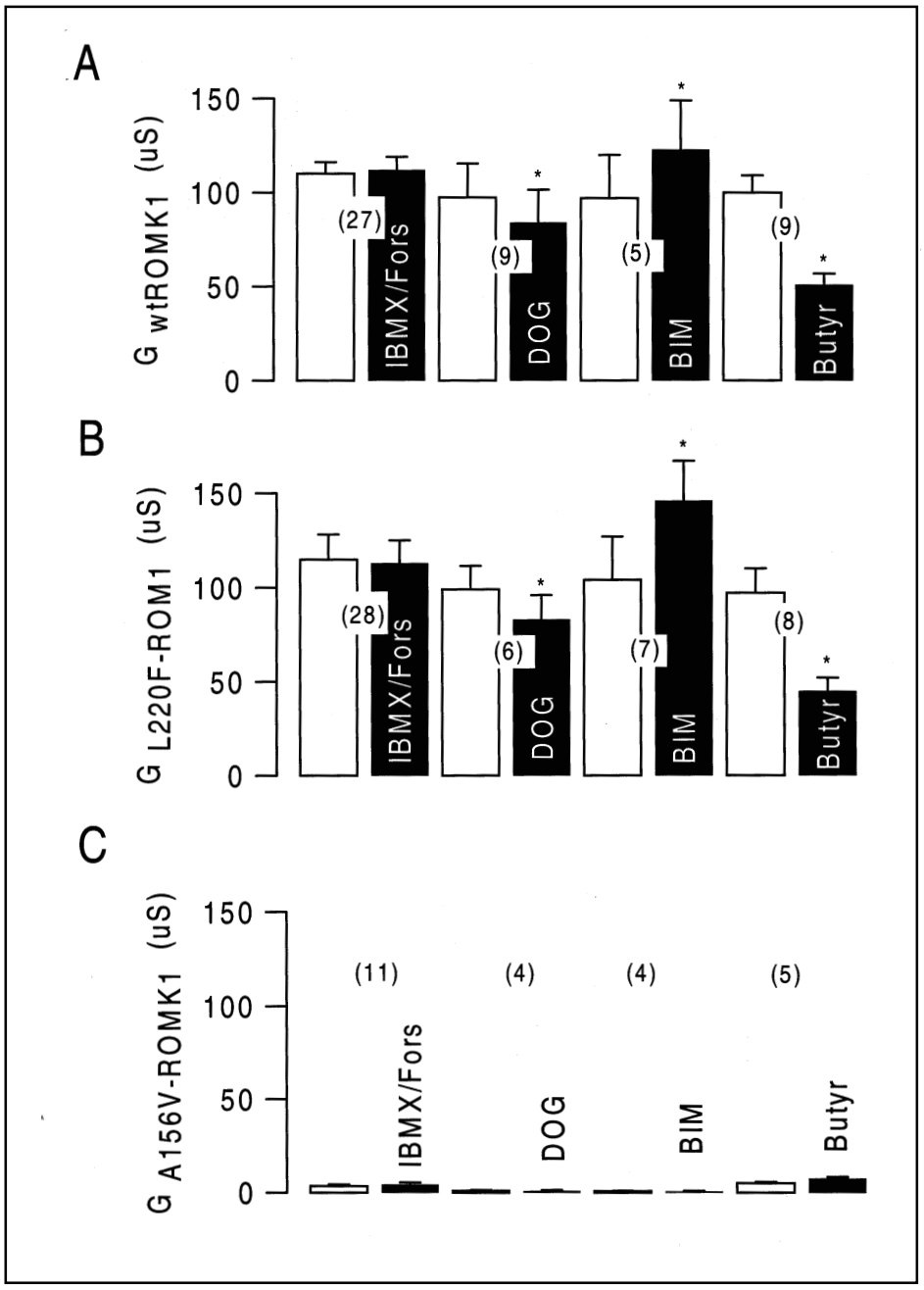

Fig. 3. Summary of the Ba^{2+}/TEA^{+} - sensitive whole cell conductances (negative current range; G_{K+}) obtained from oocytes expressing either wt-ROMK1 (A), L220F-ROMK1 (B) or A156V-ROMK1 (C). Conductances were assessed before (white bars) and after (black bars) exposure of the oocytes to either IBMX/forskolin (1 mmol/l / 2 µmol/l), DOG (5 µmol/l), BIM (10 nmol/l) or Butyrate (3 mmol/l, pH 6.5). Means ± SEM (number of experiments). Asterisks indicate significant difference from control.

coexpressing A156V-ROMk1 with L220F-ROMK1. As shown in Fig. 4A, coexpression of both ROMK1 mutants did not induce any detectable K^{+} current. The same was true when A156V-ROMK1 was coexpressed together with wt-ROMK1 at a 1:1 ratio (Fig. 4B). These data suggest that A156V-ROMK1 exerts an inhibitory effect on both L220F-ROMK1 and wt-ROMK1. When the amount of cRNA for wt-ROMK1 was enhanced relatively to that of A156V-ROMK1, a K^{+} conductance was regained supporting the idea of a dominant negative effect of A156V-ROMK1 due to formation of heteropolymeric channels. It should be mentioned that coexpression of L220F-ROMK1 with wt-ROMK1 did not exert any suppressive effects on wt-ROMK1, i.e. currents obtained in wt-ROMK1 and L220F-ROMK1/wt-ROMK1 coexpressing oocytes were comparable (data not shown).

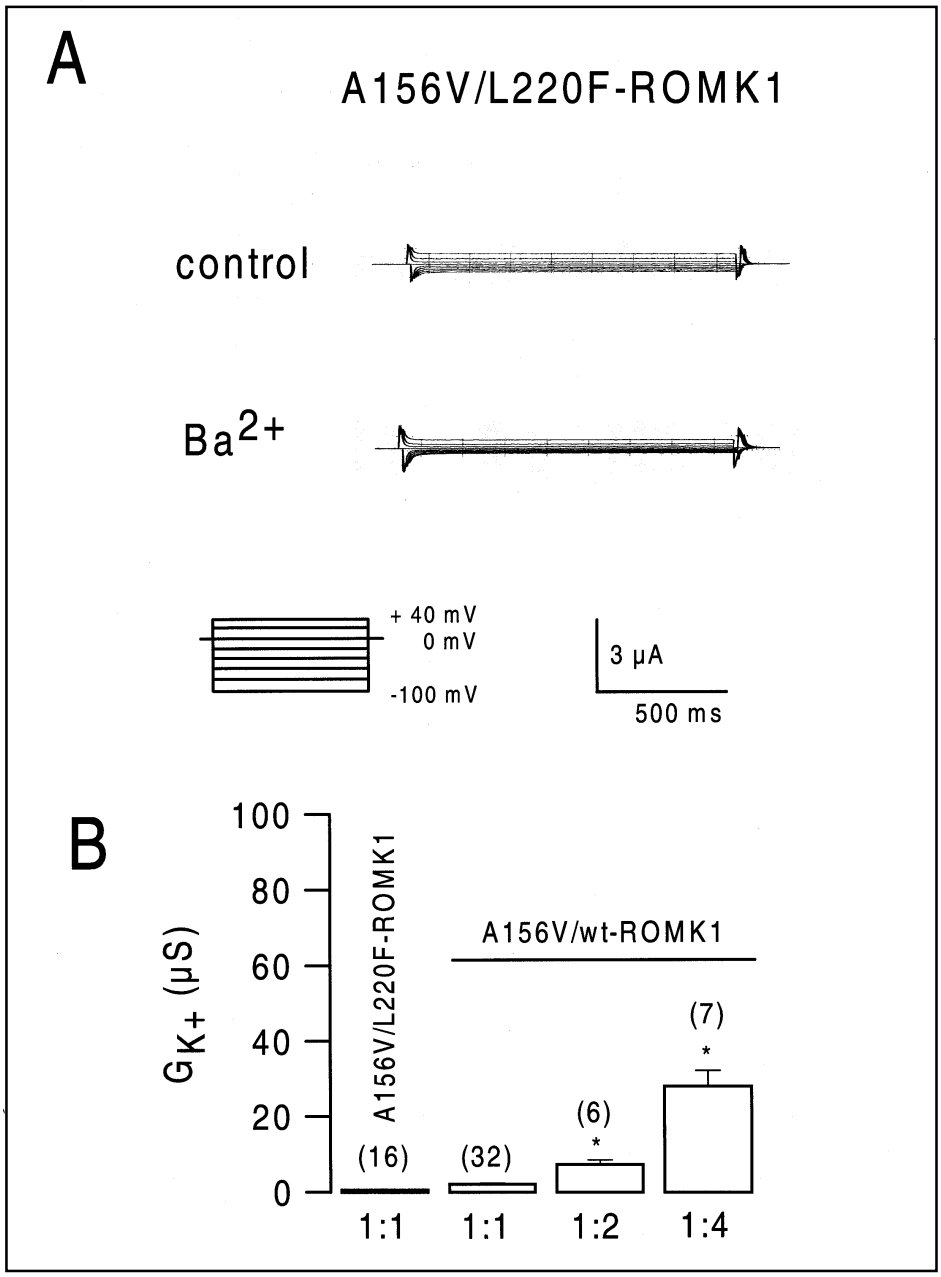

Fig. 4. Coexpression of A156V-ROMK1 with L220F-ROMK1 or A156V-ROMK1 with wt-ROMK1 in *Xenopus* oocytes. A) Representative example of whole cell currents produced by A156V-ROMK1/L220F-ROMK1 before and after inhibition by Ba^{2+} and TEA^{+} (5 mM each). B) Summary of the Ba^{2+}/TEA^{+} - sensitive whole cell conductances (negative current range; G_{K+}). cRNA of A156V-ROMK1 was coinjected with wt-ROMK1-cRNA in various ratios (1:1, 1:2, 1:4). Means ± SEM (number of experiments). Asterisks indicate significant difference from control.

Discussion

Two novel mutations in the ROMK1 K^{+} channel protein were detected recently in a patient with typical antenatal Bartter's syndrome [5]. The location of these mutations within the putative channel topology imply different effects on K^{+} channel function and regulatory properties: A156 is located within the extracellular H5 loop and is likely to interfere with formation of the channel pore, whilst L220 is adjacent to the putative PKC phosphorylation site and close to a PKA phosphorylation sites and thus may affect protein kinase dependent regulation of ROMK1 [9;13]. Moreover, another mutation (S219R) located close to L220 did abolish channel activity in another study [14]. However, as a rather unexpected result, expression of L220F-ROMK1 induced K^{+} currents in *Xenopus* oocytes comparable to that of wt-ROMK1. In contrast, A156V-ROMK1 did not induce any K^{+} conductance which was somehow expected regarding the location of A156V within the H5 loop and given the hypothesis that ROMK1 functions as a tetrameric protein [15]. This results is also consistent with prior studies on

other mutations located in close proximity to A156 [16;17].

We attempted to characterize both wt-ROMK1 and mutant ROMK1 by expression in *Xenopus* oocytes. We found no effects of intracellular cAMP/PKA on ROMK1. The apparent lack of PKA – dependent regulation of ROMK1 in *Xenopus* oocytes is likely to be caused by a missing A kinase anchoring protein that is required for the mediation of PKA effects on ROMK1 [18]. In contrast, ROMK1 remains sensitive towards PKC when expressed in *Xenopus oocytes*. Inhibition of ROMK1 by PKC has been described in earlier reports [19;20]. The rather weak effects detected here, however, were comparable in both wt-ROMK1 and the mutant L220F-ROMK1 and were not seen in A156V-ROMK1 expressing oocytes. Apart from the effects of PKA and PKC we also examined effects of an increase in intracellular Ca^{2+} on wt-ROMK1 and mutant ROMK1, but did not see any change in ROMK1 activity (data not shown), thereby confirming results of previous studies [20]. Finally, acidification of oocytes by applying 3 mmol/l butyrate at pH of 6.5 inhibited wt-ROMK1 and L220F-ROMK1 to similar degrees. We conclude that the mutation L220F does not interfere at least with the regulatory properties of ROMK1 expressed in *Xenopus* oocytes.

Previous analysis of other Bartter's causing mutations in ROMK1 demonstrated abortion of channel function for all these mutations [3;21]. In fact, also A156V-ROMK1 did not induce any detectable K^+ channel activity in *Xenopus* oocytes and was not influenced by any of the maneuvers described above. Moreover, coexpression experiments show a clear dominant negative effect of A156V-ROMK1 on both L220F-ROMK1 and wt-ROMK1. Dominant negative suppression of L220F-ROMK1 currents in the formerly described female patient is probably the cause for manifestation of the neonatal Bartter's syndrome [5]. The finding of a dominant negative effect of A156V-ROMK1 poses some questions regarding the inheritance pattern in a family, in which both mutations occurred in a compound heterozygous form [5]. If the disease mechanism in this family is based on the dominant negative effect of A156V, the father, who also carries this mutation in the heterozygous state would have to show symptoms of Bartter's syndrome. Although it is rather unlikely that symptoms in the father were overlooked, no further clinical data are available. In addition, no example of autosomal dominant inheritance has been reported in Bartter's syndrome so far. A possible explanation of this discrepancy might be that the dominant negative effect of A156V-ROMK1 on wt-ROMK1 demonstrated here does not occur *in vivo*, while the dominant negative effect on L220F-ROMK1 is expressed, thus explaining loss of function in the compound heterozygote child. In fact, the anchoring protein dependent kinase A phosphorylation that is present in the native tissue but is missing in *Xenopus* oocytes might be a good candidate [18] for this suppressive effect. The data presented here do not clearly discriminate whether dominant effects are caused by affecting maturation of the protein or whether it is due to abortion of putative heteropolymeric channel function. However, expression of another unrelated K^+ channel, K_VLQT1, was not affected by coexpression of A156V-ROMK1. Therefore, some of the experiments described here, including those shown in Fig. 4B, favor hypothesis of a defective heterotetrameric channel.

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